EXPRESSION OF G PROTEIN COUPLED RECEPTORS IN YEAST

This invention was made with government support under NIH grants HL16037 and GM21841. The government may have certain rights to this invention.

Field of the Invention

This invention relates to yeast cells expressing heterologous G protein coupled receptors, vectors useful for making such cells, and methods of using the same.

Background of the Invention

The actions of many extracellular signals (for example, neurotransmitters, hormones, odorants, light) are mediated by receptors with seven transmembrane domains (G protein coupled receptors) and heterotrimeric guanine nucleotide-binding regulatory proteins (G proteins). See H. Dohlman, M. Caron, and R. Lefkowitz, Biochemistry 26, 2657 (1987); L. Stryer and H. Bourne, Ann. Rev. Cell Biol. 2, 391 (1988). Such G protein-mediated signaling systems have been identified in organisms as divergent as yeast and man. See H. Dohlman et al., supra; L. Stryer and H. Bourne, supra; K. Blumer and J. Thorner, Annu.Rev. Physiol. (in press). The β2-adrenergic receptor (βAR) is the prototype of the seven-transmembrane-segment class of

ligand binding receptors in mammalian cells. In response to epinephrine or norepinephrine, βAR activates a G protein, G_s, which in turn stimulates adenylate cyclase and cyclic adenosine monophosphate production in the cell. See H. Dohlman et al., supra; L. Stryer and H. Bourne, supra. G protein-coupled pheromone receptors in yeast control a developmental program that culminates in mating (fusion) of a and α haploid cell types to form the a/α diploid. See K. Blumer and J. Thorner, supra; I. Herskowitz, Microbiol. Rev. 52, 536 (1988).

The present invention is based on our continued research into the expression of heterologous G protein coupled receptors in yeast.

Summary of the Invention

A first aspect of the present invention is a transformed yeast cell containing a first heterologous DNA sequence which codes for a mammalian G protein coupled receptor and a second heterologous DNA sequence which codes for a mammalian G protein α subunit (mammalian G_α). The first and second heterologous DNA sequences are capable of expression in the cell, but the cell is incapable of expressing an endogenous G protein α-subunit (yeast G_α). The cell optionally contains a third heterologous DNA sequence, with the third heterologous DNA sequence comprising a pheromone-responsive promotor and an indicator gene positioned downstream from the pheromone-responsive promoter and operatively associated therewith.

A second aspect of the present invention is a method of testing a compound for the ability to affect the rate of dissociation of G_α from $G_{\beta\gamma}$ in a cell. The method comprises: providing a transformed yeast cell as described above; contacting the compound to the cell; and then detecting the rate of dissociation of G_α from $G_{\beta\gamma}$ in the cell. The cells may be provided in an

aqueous solution, and the contacting step carried out by adding the compound to the aqueous solution.

A third aspect of the present invention is a DNA expression vector capable of expressing a transmembrane protein into the cell membrane of yeast cells. The vector contains a first segment comprising at least a fragment of the extreme amino-terminal coding sequence of a yeast G protein coupled receptor. A second segment is positioned downstream from the first segment (and in correct reading frame therewith), with the second segment comprising a DNA sequence encoding a heterologous G protein coupled receptor.

A fourth aspect of the present invention is a yeast cell transformed by a vector as described above.

Brief Description of the Drawings

Figure 1 illustrates the construction of the yeast human β2 Adrenergic Receptor expression plasmid, pYβAR2.

Figure 2 illustrates hβAR ligand binding to membranes from pYβAR2-transformed yeast cells.

Figure 3 shows a comparison of β-adrenergic agonist effects on pheromone-inducible gene activity. α-MF, 10 μM α-mating factor; (-) ISO, 50 μM (-) isoproterenol; (-) ALP, 50 μM (-) alprenolol; (+) ISO, 100 μM (+) isoproterenol.

Detailed Description of the Invention

Nucleotide bases are abbreviated herein as follows:

A=Adenine	G=Guanine
C=Cytosine	T=Thymine

Amino acid residues are abbreviated herein to either three letters or a single letter as follows:

Ala;A=Alanine	Leu;L=Leucine
Arg;R=Arginine	Lys;K=Lysine
Asn;N=Asparagine	Met;M=Methionine
Asp;D=Aspartic acid	Phe;F=Phenylalanine
Cys;C=Cysteine	Pro;P=Proline

Gln;Q=Glutamine	Ser;S=Serine
Glu;E=Glutamic acid	Thr;T=Threonine
Gly;G=Glycine	Trp;W=Tryptophan
His;H=Histidine	Tyr;Y=Tyrosine
Ile;I=Isoleucine	Val;V=Valine

The term "mammalian" as used herein refers to any mammalian species (e.g., human, mouse, rat, and monkey).

The term "heterologous" is used herein with respect to yeast, and hence refers to DNA sequences, proteins, and other materials originating from organisms other than yeast (e.g., mammalian, avian, amphibian), or combinations thereof not naturally found in yeast.

The terms "upstream" and "downstream" are used herein to refer to the direction of transcription and translation, with a sequence being transcribed or translated prior to another sequence being referred to as "upstream" of the latter.

G proteins are comprised of three subunits: a guanyl-nucleotide binding α subunit; a β subunit; and a τ subunit. G proteins cycle between two forms, depending on whether GDP or GTP is bound thereto. When GDP is bound the G protein exists as an inactive heterotrimer, the $G_{\alpha\beta\tau}$ complex. When GTP is bound the α subunit dissociates, leaving a $G_{\beta\tau}$ complex. Importantly, when a $G_{\alpha\beta\tau}$ complex operatively associates with an activated G protein coupled receptor in a cell membrane, the rate of exchange of GTP for bound GDP is increased and, hence, the rate of dissociation of the bound the α subunit from the $G_{\beta\tau}$ complex increases. This fundamental scheme of events forms the basis for a multiplicity of different cell signaling phenomena. See generally Stryer and Bourne, supra.

Any mammalian G protein coupled receptor, and the DNA sequences encoding these receptors, may be employed in practicing the present invention. Examples

of such receptors include, but are not limited to, dopamine receptors, muscarinic cholinergic receptors, α-adrenergic receptors, β-adrenergic receptors, opiate receptors, cannabinoid receptors, and serotonin receptors. The term receptor as used herein is intended to encompass subtypes of the named receptors, and mutants and homologs thereof, along with the DNA sequences encoding the same.

The human D_1 dopamine receptor cDNA is reported in A. Dearry et al., <u>Nature</u> **347**, 72-76 (1990).

The rat D_2 dopamine receptor cDNA is reported in J. Bunzow et al., <u>Nature</u> **336**, 783-787 (1988); <u>see also</u> O. Civelli, et al., PCT Appln. WO 90/05780 (all references cited herein are to be incorporated herein by reference).

Muscarinic cholinergic receptors (various subtypes) are disclosed in E. Peralta et al., <u>Nature</u> **343**, 434 (1988) and K. Fukuda et al., <u>Nature</u> **327**, 623 (1987).

Various subtypes of α_2-adrenergic receptors are disclosed in J. Regan et al., <u>Proc. Natl. Acad. Sci. USA</u> **85**, 6301 (1988) and in R. Lefkowitz and M. Caron, <u>J. Biol. Chem.</u> **263**, 4993 (1988).

Serotonin receptors (various subtypes) are disclosed in S. Peroutka, <u>Ann. Rev. Neurosci.</u> **11**, 45 (1988).

A cannabinoid receptor is disclosed in L. Matsuda et al., <u>Nature</u> **346**, 561 (1990).

Any DNA sequence which codes for a mammalian G α subunit (G_α) may be used to practice the present invention. Examples of rammalian G α subunits include G_s α subunits, G_i α subunits, G_o α subunits, G_z α subunits, and transducin α subunits. <u>See generally</u> Stryer and Bourne, <u>supra</u>. G proteins and subunits useful for practicing the present invention include subtypes, and mutants and homologs thereof, along with the DNA sequences encoding the same.

Heterologous DNA sequences are expressed in a host by means of an expression vector. An expression vector is a replicable DNA construct in which a DNA sequence encoding the heterologous DNA sequence is operably linked to suitable control sequences capable of effecting the expression of a protein or protein subunit coded for by the heterologous DNA sequence in the intended host. Generally, control sequences include a transcriptional promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and (optionally) sequences which control the termination of transcription and translation.

Vectors useful for practicing the present invention include plasmids, viruses (including phage), and integratable DNA fragments (i.e., fragments integratable into the host genome by homologous recombination). The vector may replicate and function independently of the host genome, as in the case of a plasmid, or may integrate into the genome itself, as in the case of an integratable DNA fragment. Suitable vectors will contain replicon and control sequences which are derived from species compatible with the intended expression host. For example, a promoter operable in a host cell is one which binds the RNA polymerase of that cell, and a ribosomal binding site operable in a host cell is one which binds the endogenous ribosomes of that cell.

DNA regions are operably associated when they are functionally related to each other. For example: a promoter is operably linked to a coding sequence if it controls the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation. Generally, operably linked means contiguous and, in the case of leader sequences, contiguous and in reading phase.

Transformed host cells of the present invention are cells which have been transformed or transfected with the vectors constructed using recombinant DNA techniques and express the protein or protein subunit coded for by the heterologous DNA sequences. In general, the host cells are incapable of expressing an endogenous G protein α-subunit (yeast G_α). The host cells do, however, express a complex of the G protein β subunit and the G protein τ subunit ($G_{\beta\tau}$). The host cells may express endogenous $G_{\beta\tau}$, or may optionally be engineered to express heterologous $G_{\beta\tau}$ (e.g., mammalian) in the same manner as they are engineered to express heterologous G_α.

A variety of yeast cultures, and suitable expression vectors for transforming yeast cells, are known. See, e.g., U.S. Patent No. 4,745,057; U.S. Patent No. 4,797,359; U.S. Patent No. 4,615,974; U.S. Patent No. 4,880,734; U.S. Patent No. 4,711,844; and U.S. Patent No. 4,865,989. Saccharomyces cerevisiae is the most commonly used among the yeast, although a number of other strains are commonly available. See, e.g., U.S. Patent No. 4,806,472 (Kluveromyces lactis and expression vectors therefor); 4,855,231 (Pichia pastoris and expression vectors therefor). Yeast vectors may contain an origin of replication from the 2 micron yeast plasmid or an autonomously replicating sequence (ARS), a promoter, DNA encoding the heterologous DNA sequences, sequences for polyadenylation and transcription termination, and a selection gene. An exemplary plasmid is YRp7, (Stinchcomb et al., Nature 282, 39 (1979); Kingsman et al., Gene 7, 141 (1979); Tschemper et al., Gene 10, 157 (1980)). This plasmid contains the trp1 gene, which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC No. 44076 or PEP4-1 (Jones, Genetics 85, 12 (1977)). The presence of the trp1 lesion in the

yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable promoting sequences in yeast vectors include the promoters for metallothionein, 3-phosphoglycerate kinase (Hitzeman et al., J. Biol. Chem. 255, 2073 (1980) or other glycolytic enzymes (Hess et al., J. Adv. Enzyme Reg. 7, 149 (1968); and Holland et al., Biochemistry 17, 4900 (1978)), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Suitable vectors and promoters for use in yeast expression are further described in R. Hitzeman et al., EPO Publn. No. 73,657. Other promoters, which have the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and the aforementioned metallothionein and glyceraldehyde-3-phosphate dehydrogenase, as well as enzymes responsible for maltose and galactose utilization.

In constructing suitable expression plasmids, the termination sequences associated with these genes may also be ligated into the expression vector 3′ of the heterologous coding sequences to provide polyadenylation and termination of the mRNA.

A novel DNA expression vector described herein which is particularly useful for carrying out the present invention contains a first segment comprising at least a fragment of the extreme amino-terminal coding sequence of a yeast G protein coupled receptor and a second segment downstream from said

first segment and in correct reading frame therewith, the second segment comprising a DNA sequence encoding a heterologous G protein coupled receptor (e.g., a mammalian G protein coupled receptor). In a preferred embodiment, this vector comprises a plasmid. In constructing such a vector, a fragment of the extreme amino-terminal coding sequence of the heterologous G protein coupled receptor may be deleted. The first and second segments are operatively associated with a promoter, such as the GAL1 promoter, which is operative in a yeast cell. Coding sequences for yeast G protein coupled receptors which may be used in constructing such vectors are exemplified by the gene sequences encoding yeast phereomone receptors (e.g., the STE2 gene, which encodes the α-factor receptor, and the STE3 gene, which encodes the a-factor receptor). The levels of expression obtained from these novel vectors are enhanced if at least a fragment of the 5'-untranslated region of a yeast G protein coupled receptor gene (e.g., a yeast pheromone receptor gene; see above) is positioned upstream from the first segment and operatively associated therewith.

Any of a variety of means for detecting the dissociation of G_α from $G_{\beta\gamma}$ can be used in connection with the present invention. The cells could be disrupted and the proportion of these subunits and complexes determined physically (i.e., by chromatography). The cells could be disrupted and the quantity of G_α present assayed directly by assaying for the enzymatic activity possessed by G_α in isolation (i.e., the ability to hydrolyze GTP to GDP). Since whether GTP or GDP is bound to the G protein depends on whether the G protein exists as a $G_{\beta\gamma}$ or $G_{\alpha\beta\gamma}$ complex, dissociation can be probed with radiolabelled GTP. As explained below, morphological changes in the cells can be observed. A particularly convenient method, however, is to provide in the cell a third heterologous

DNA sequence, wherein the third heterologous DNA sequence comprises a pheromone-responsive promotor and an indicator gene positioned downstream from the pheromone-responsive promoter and operatively associated therewith. This sequence can be inserted with a vector, as described in detail herein. With such a sequence in place, the detecting step can be carried out by monitoring the expression of the indicator gene in the cell. Any of a variety of pheromone responsive promoters could be used, examples being the BAR1 gene promoter and the FUS1 gene promoter. Likewise, any of a broad variety of indicator genes could be used, with examples including the HIS3 gene and the LacZ gene.

As noted above, transformed host cells of the present invention express the protein or protein subunit coded for by the heterologous DNA sequence. When expressed, the G protein coupled receptor is located in the host cell membrane (i.e., physically positioned therein in proper orientation for both the stereospecific binding of ligands on the extracellular side of the cell membrane and for functional interaction with G proteins on the cytoplasmic side of the cell membrane).

The ability to control the yeast pheromone response pathway by expression of a heterologous adrenergic receptor and its cognate G protein α-subunit has the potential to facilitate structural and functional characterization of mammalian G protein-coupled receptors. By scoring for responses such as growth arrest or β-galactosidase induction, the functional properties of mutant receptors can now be rapidly tested. Similarly, as additional genes for putative G protein-coupled receptors are isolated, numerous ligands can be screened to identify those with activity toward previously unidentified receptors. See F. Libert et al., Science 244, 569 (1989); M. S. Chee

et al., Nature 344, 774 (1990). Moreover, as additional genes coding for putative G protein α-subunits are isolated, they can be expressed in cells of the present invention and screened with a variety of G protein coupled receptors and ligands to characterize these subunits. These cells can also be used to screen for compounds which affect receptor-G protein interactions.

Cells of the present invention can be deposited in the wells of microtiter plates in known, predetermined quantities to provide standardized kits useful for screening compounds in accordance with the various screening procedures described above.

The following Examples are provided to further illustrate various aspects of the present invention. They are not to be construed as limiting the invention.

EXAMPLE 1

Construction of the Human β2-Adrenergic Expression Vector pYβAR2 and Expression in Yeast

To attain high level expression of the human β2-adrenergic receptor (hβAR) in yeast, a modified hβAR gene was placed under the control of the GAL1 promoter in the multicopy vector, YEp24 (pYβAR2).

Figure 1 illustrates the construction of yeast expression plasmid pYβAR2. In pYβAR2, expression of the hβAR sequence is under the control of the GAL1 promoter. Figure 1A shows the 5'-untranslated region and the first 63 basepairs (bp) of coding sequence of the hβAR gene in pTZNAR, B. O'Dowd et al., J. biol. Chem. 263, 15985 (1988), which was removed by Aat II cleavage and replaced with a synthetic oligonucleotide corresponding to 11 bp of noncoding and 42 bp of coding sequence from the STE.2 gene. See N. Nakayama et al., EMBO J. 4, 2643 (1985); A. Burkholder and L. Hartwell, Nucleic Acids Res. 13, 8463 (1985). The resulting

plasmid, pTZYNAR, contains the modified hβAR gene flanked by Hind III sites in noncoding sequences. The Hind III-Hind III fragment was isolated from pTZYNAR and inserted into pAAH5 such that the 3′- untranslated sequence of the modified hβAR gene was followed by 450 bp containing termination sequences from the yeast ADH1 gene. See G. Ammerer, Methods. Enzymol. 101, 192 (1983).

As illustrated in Figure 1B, pyβ13AR2 was constructed by inserting the Bam HI - Bam HI fragment containing hβAR and ADJ1 sequences into YEpG24. E. Wyckoff and T. Hsieh, Proc. Natl. Acad. Sci. U.S.A. 85, 6272 (1988). Where maximum expression was sought, cells were cotransformed with plasmid pMTL9 (from Dr. S. Johnston) containing LAC9, a homolog of the S. cerevisiae GAL4 transactivator protein required for GAL1-regulated transcription. J. Salmeron et al., Mol. Cell. Biol. 9, 2950 (1989). Cells grown to late exponential phase were induced in medium containing 3% galactose, supplemented with about 10 μM alprenolol, and grown for an additional 36 hours. Standard methods for the maintenance of cells were used. See F. Sherman et al., Methods in Yeast Genetics (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1986).

Maximal expression required (i) expression of a transcriptional transactivator protein (LAC9), (ii) replacement of the 5′ untranslated and extreme NH_2-terminal coding sequence of the hβAR gene with the corresponding region of the yeast STE2 (α-factor receptor) gene, (iii) induction with galactose when cell growth reached late exponential phase, and, (iv) inclusion of an adrenergic ligand in the growth medium during induction.

The plasmid pYβAR2 was deposited in accordance with the provisions of the Budapest Treaty at the American Type Culture Collection, 12301 Parklawn

Drive, Rockville, MD 20852 USA, on September 11, 1990, and has been assigned ATCC Accession No. 40891.

EXAMPLE 2

Binding Affinity of hβAR Ligands in Yeast Transformed with pYβAR2

A primary function of cell surface receptors is to recognize only appropriate ligands among other extracellular stimuli. Accordingly, ligand binding affinities were determined to establish the functional integrity of hβAR expressed in yeast. As discussed in detail below, an antagonist, ^{125}I-labeled cyanopindolol (^{125}I-CYP), bound in a saturable manner and with high affinity to membranes prepared from pYβAR2-transformed yeast cells. By displacement of ^{125}I-CYP with a series of agonists, the order of potency and stereospecificity expected for hβAR was observed.

SC261 cells (MATa ura3-52 trpl leu2 prbl-1122 pep4-3 prcl-407) (from Dr. S. Johnston) harboring pYβAR2 (URA3) and pMTL9 (LEU2) were grown in minimal glucose-free selective media to late log phase (OD_{600} = 5.0), and then induced with the addition of 3% galactose and 40 μM alprenolol. After 36 hours, cells were harvested and spheroplasts were prepared as described. See E. Wyckoff and T. Hsieh, Proc. Natl. Acad. Sci. U.S.A. 85, 6272 (1988). Briefly, the spheroplasts were resuspended in 50 mM Tris-HCl pH 7.4, 5 mM EDTA and were lysed by vortex mixing with glass beads for three one-min periods at 4°C. Crude membranes were prepared from the lysates and binding assays with ^{125}I-CYP were performed by methods described previously. See H. Dohlman et al., Biochemistry 29, 2335 (1990).

Figure 2 illustrates hβAR ligand binding to membranes from pYβAR2-transformed yeast cells. (A) B_{max} (maximum ligand bound) and K_d (ligand dissociation constant) values were determined by varying 125_I-CYP concentrations (5 - 400 pM). Specific binding was

defined as the amount of total binding (circles) minus nonspecific binding measured in the presence of 10 μM (-) alprenolol (squares). A K_d of 93 pM for 125_I-CYP binding was obtained and used to calculate agonist affinities (below). (B) Displacement of 18 pM 125_I-CYP with various concentrations of agonists was used to determine apparent low affinity K_I values (non G protein coupled, determined in the presence of 50 μM GTP) for receptor binding, squares; (-) isoproterenol, circles; (-) epinephrine, downward-pointing triangles; (+) isoproterenol, upward pointing triangles; (-) norepinephrine.

COMPARATIVE EXAMPLE A

Ligand Binding Affinity for hβAR Expressed in Yeast and Mammalian Cells

The binding data of Figures 2 (A) and (B) were analyzed by nonlinear least squares regression, see A. DeLean et al., Mol. Pharmacol. 21, (1982), and are presented in Table I. Values given are averages of measurements in triplicate, and are representative of 2 - 3 experiments. Binding affinities in yeast were nearly identical to those observed previously for hβAR expressed in mammalian cells.

Table 1

Comparison of ligand Binding Parameters for High Level Expression of Human β-Adrenergic Receptor in Yeast and COS-7 Cells*

	Yeast SC261 (pY,βAR2, pMTL9)	Monkey COS-7 (pBC12:β,BAR)
$^{125}_{1}$-CYP:		
$^{1}K_d$	0.093 nM ± 0.013	0.110 nM ±0.009
$^{2}B_{max}$	115 pmol/mg	24 pmol/mg
$^{3}K_i$(M):		
(-) isoproterenol	103 ± 26	130 ± 15
(+) isoproterenol	3670 ± 420	4000 ± 184
(-) epinephrine	664 ± 123	360 ± 30
(-) norepinephrine	6000 ± 1383	5800 ± 373

*Values derived from Fig. 2 and H. Dohlman et al., Biochemistry 29, 2335 (1990).; ± S.E.
$^{1}K_d$, ligand dissociation constant
$^{2}B_{max}$, maximum ligand bound
$^{3}K_i$, inhibition constant

EXAMPLE 3

Agonist-Dependent Activation of Mating Signal Transduction in Yeast Expressing hβAR

A second major function of a receptor is agonist-dependent regulation of downstream components in the signal transduction pathway. Because the pheromone-responsive effector in yeast is not known, indirect biological assays are the most useful indicators of receptor functionality. See K. Blumer and J. Thorner, Annu.Rev. Physiol. in press; I. Herskowitz, Microbiol. Rev. 52, 536 (1988). In yeast

cells expressing high concentrations of hβAR, no agonist-dependent activation of the mating signal transduction pathway could be detected by any of the typical in vivo assays; for example, imposition of G1 arrest, induction of gene expression, alteration of morphology (so-called "shmoo" formation) or stimulation of mating. A likely explanation for the absence of responsiveness is that hβAR was unable to couple with the endogenous yeast G protein.

EXAMPLE 4

Coexpression of hβAR and Mammalian G_s α-Subunit in Yeast

Expression of a mammalian G_s α-subunit can correct the growth defect in yeast cells lacking the corresponding endogenous protein encoded by the GPA1 gene. See C. Dietzel and J. Kurjan, Cell 50, 1001 (1987). Moreover, specificity of receptor coupling in mammalian cells is conferred by the α-subunit of G proteins. See L. Stryer and H. Bourne, Annu. Rev. Cell Biol. 2, 391 (1988). Thus, coexpression of hβAR and a mammalian G_s α-subunit (GSα) in yeast was attempted to render the yeast responsive to adrenergic ligands. Accordingly, a cDNA encoding rat $G_s\alpha$ under the control of the copper-inducible CUP1 promoter was introduced on a second plasmid, pYSK136Gαs. See C. Dietzel and J. Kurjan, Cell 50, 1001 (1987). In yeast (NNY19) coexpressing hβAR and rat $G_s\alpha$, but containing wild-type GPA1, no adrenergic agonist-induced shmoo formation, a characteristic morphological change of yeast in response to mating pheromone, was observed.

EXAMPLE 5

Coexpression of hβAR and Mammalian G_sα-Subunit in Yeast Lacking an Endogenous G Protein α-Subunit

To prevent interference by the endogenous yeast G protein α-subunit, gpa1 mutant cells (strain

8c) were used.

Yeast strain 8c (MATa ura3 leu2 his3 trp1 gpa1::H153), I. Miyajima et al., Cell 50, 1011 (1987), carrying plasmids pYSK136Gαs (TRP1), C. Dietzel and J. Kurjan, Cell 50, 1001 (1987), pMTL9 (LEU2), J. Salmeron et al., Mol. Cell. Biol. 9, 2950 (1989), and pYβAR2 (URA3) was maintained on glucose-free minimal selective plates containing 3% glycerol, 2% lactic acid, 50 μM $CuSO_4$ and 3% galactose. Colonies were transferred to similar plates containing 0.5 mM ascorbic acid and the indicated adrenergic ligand(s). After 16-20 hours at 30°C, the colonies were transferred to similar liquid media at a density of 10^6-10^7 cells/ml and examined by phase contrast microscopy.

Morphologies of yeast cells cotransformed with pYβAR2, pMTL9, and pYSK136Gαs were examined after incubation with (A) no adrenergic agent; (B) 100 μM (-) isoproterenol; (C) 100 μM (-) isoproterenol and 50 μM (-) alprenolol; and (D) 100 μM (+) isoproterenol. Results showed that treatment of 8c cells coexpressing hβAR and rat $G_s\alpha$ with the β-adrenergic agonist isoproterenol indeed induced shmoo formation, and that this effect was blocked by the specific antagonist alprenolol.

EXAMPLE 6

Coexpression of hβAR and Mammalian $G_s\alpha$-Subunit in Yeast Containing a β-Galactosidase Signal Sequence

The isoproterenol-induced morphological response of 8c cells coexpressing hβAR and rat $G_s\alpha$ suggested that these components can couple to each other and to downstream components of the pheromone response pathway in yeast lacking the endogenous G α-subunit. To confirm that the pheromone signaling pathway was activated by hβAR and rat $G_s\alpha$, agonist induction of the pheromone-responsive FUS1 gene promoter was measured in a strain of yeast derived from

8c cells (8c1) in which a FUS1-lacZ gene fusion had been stably integrated into the genome. See S. Nomoto et al., EMBO J. 9, 691 (1990).

Strains 8c (Fig. 3, legend) and NNY19 (MATa ura3 leu2 his3 trp1 lys2 FUS1-LacZ::LEU2) were modified by integrative transformation with YIpFUS102 (LEU2), S. Nomoto et al., supra, and designated 8c1 and NNY19, respectively. These strains were transformed with pYβAR2 and pYSK136Gαs and maintained on minimal selective plates containing glucose and 50 μM $CuSO_4$. Colonies were inoculated into minimal selective media (3% glycerol, 2% lactic acid, 50 μM $CuSO_4$), grown to early log phase (OD_{600} = 1.0), and induced for 12 hours by addition of 3% galactose. Cells were washed and resuspended in induction media (OD_{600} = 5.0) containing 0.5 mM ascorbic acid and the indicated ligands. After a 4 hour incubation at 30°C, cells were harvested, resuspended into 1 ml of Z-buffer, see J. Miller, Experiments in Molecular Genetics (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1972), supplemented with 0.0075% SDS, and β-galactosidase activities were determined in 3 - 4 independent experiments as described previously. See J. Miller, supra.

Figure 3 shows a comparison of β-adrenergic agonist effects on pheromone-inducible gene activity. α-MF, 10 μM α-mating factor; (-) ISO, 50 μM (-) isoproterenol; (-) ALP, 50 μM (-) alprenolol; (+) ISO, 100 μM (+) isoproterenol. In 8c1 (gpa1) cells coexpressing hβAR and rat $G_s\alpha$, a dramatic isoproterenol-stimulated induction of β-galactosidase activity was observed. Agonist stimulation was stereoselective and was blocked by addition of a specific antagonist. Agonist responsiveness was dependent on expression of both hβAR and rat $G_s\alpha$, and required a strain in which the endogenous G protein α-subunit was disrupted. The final β-galactosidase activity achieved in response to isoproterenol in transformed 8c1 cells was comparable

to that induced by α-factor in nontransformed cells that express GPA1 (NNY19), although basal β-galactosidase activity in NNY19 cells was considerably lower than in 8c1 cells. Taken together, our results indicated that coexpression of hβAR and rat $G_s\alpha$ was sufficient to place under catecholamine control key aspects of the mating signal transduction pathway in yeast. However, the adrenergic agonist did not stimulate mating in either 8c cells or NNY19 cells coexpressing hβAR and rat $G_s\alpha$, in agreement with recent observations that yeast pheromone receptors, in addition to binding pheromones, participate in other recognition events required for mating. See A. Bender and G. Sprague, Genetics 121, 463 (1989).

hβAR stimulates adenylate cyclase in animal cells via the action of the α-subunit of its G protein. In contrast, mating factor receptors in yeast trigger their effector via the action of the $\beta\gamma$ subunits. M. Whiteway et al., Cell 56, 476 (1989). Our present results indicate that activation of hβAR in yeast leads to dissociation of mammalian $G_s\alpha$ from yeast $\beta\gamma$, and it is the $\beta\gamma$ subunits that presumably elicit the response.

The foregoing examples are illustrative of the present invention, and are not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.